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(54) Title: 16-HYDROXYTRIPTOLIDE COMPOSITION AND METHOD FOR IMMUNOTHERAPY

(57) Abstract

A composition for use in immunosuppression therapy is disclosed. The composition includes an immunosuppressant drug, such as cyclosporin A and an amount of 16-hydroxytriptolide effective to potentiate the immunosuppressive effect of the immunosuppressant drug. The composition is particularly useful for in treating transplantation rejection or autoimmune disease. Also disclosed is a method of immunosuppression that includes administering to a subject (i) a pharmaceutically effective amount of an immunosuppressant drug and (ii) purified 16-hydroxytriptolide in an amount effective to potentiate the action of the drug.

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16-HYDROXYTRIPTOLIDE COMPOSITION AND METHOD FOR IMMUNOTHERAPY

1. Field of the Invention

The present invention relates to a composition and method for transplantation rejection and immunosuppression.

2. References

Abbas, A.K., *et al.*, Cellular and Molecular Immunology, W.B. Sanders Co. (1991).

Altomonte, L., *et al.*, Clin. Rheumatology, 11(2):202 (1992).

Boyum., A., Scan J. Lab Invest, 21:77 (1968).

Brahn, E., *et al.*, Lymphokine and Cytokine Res. 11(5):253 (1992).

Brennan F.M., *et al.*, British Journal of Rheumatology, 31(5):293-8. (1992)

Briggs J.D., Immunology Letters Jul 29(1-2):89-94 (1991).

Brozik, M., *et al.*, J. Rheumatology, 19(1):63 (1992).

Catalano, M.A., J. Cellular Biochemistry Supplement 17B:128 (1993)

Chen, K., *et al.*, J. Nat Products, 55(1):88 (1992).

Chin, J.E., *et al.*, Arthritis and Rheumatism 33:1776-1786 (1990).

Chu, C.Q., *et al.*, British J. Rheumatology 31(10):653-661 (1992).

Danis, V.A., *et al.*, Ann. Rheumatic Diseases, 51(8):946 (1992).

Dasgupta, B., *et al.*, J. Rheumatology, 19(1):22 (1992).

DeBennedetti, F., *et al.*, Arth. and Rheumat., 34(9):1158 (1991).

DeBennedetti, F., *et al.*, Clin. & Exp. Rheumatol., 10(5):493 (1992).

Deleuran, B.W., *et al.*, Arth. and Rheumat., 35(10):1180 (1992).

Elliott, M.J., et. al., J. Cellular Biochemistry Supplement 17B: 145 (1993).

Falus, A., *et al.*, Scand. J. Rheumatol. 21(3):116 (1992).

Gilman, S.C., *et al.*, Arthritis and Rheumatism 31:126-130 (1988).

Green, L.M., *et al.*, J. Immunol Methods 70:257 (1984).

Guerne, P.A., *et al.*, J. Clin. Invest. 83:585-592 (1989).

Harigai, M., *et al.*, J. Clin. & Lab. Immun. 34:107-113 (1991).

Hasan, R., *et al.*, Transplantation 54:408 (1992).

Holt, I., *et al.*, Eur. J. Clin. Invest., 21(5):479 (1991).

Holt, I., *et al.*, Brit. J. Rheumatology, 21(11):725 (1992).

Keown, P.A., Ann. Rev. Trans., Clin. Transplants 205-223, (1991).

Kishimoto, T., J. Autoimmunity, 5 Supp. A:123 (1992).

Koch, A.E., *et al.*, Clin. Immunol. & Immunopath. 65:23-29 (1992).

Li, X. W., *et al.*, Transplantation 50:82 (1990).

McCachren, S.S., *et al.*, Arthritis and Rheumatism 32:1539-1545 (1989).

Mishell, B., et *al.*, eds., Selected Methods in Cellular Immunology, Freeman and Co. (1980).

Murase, N., et *al.*, Transplantation 55:701 (1993).

Noelle, R.J., *et al.*, FASEB 5(13):2770 (1991).

O'Gara, A. and Defrance, T.: "Bioassays for interleukins", In Laboratory Methods in Immunology, H. Zola, Ed. CRC Press (1990).

O'Neill, L.A., *et al.*, European J. Pharmacol. 166:131-137 (1989).

Platt, J.L., *et al.*, Immunol. Today 11(2):450 (1990).

Roberts, J.P., *et al.*, Ann. Rev. Med., 40:287 (1989).

Roitt, I., Essential Immunology, 7th edition, Blackwell Sci Pub, p. 63 (1991).

Sawada, T., *et al.*, Clin. and Exp. Rheumatology 9(4):363 (1991).

Schumacher, H.R. ed., Pimer on the Rheumatic Diseases, Ninth edition, Arthritis Foundation, Atlanta, GA (1988).

Smith, J.B., *et al.*, Rheumatology Int'l 9(2):53-58 (1989).

Storb, R., "Pathophysiology and Prevention of Graft-Versus-Host Disease," in Advances in Immunobiology: Blood Cell Antigens and Bone Marrow Transplantation, McCullogh, J., and Sandler, S.G., editors, Alan R. Liss, Inc., New York, p. 337 (1984).

Tan, P.L., *et al.*, J. Rheumatol., 17(12):1608 (1990).

Thompson, R.C., *et al.*, Int'l J. Immunopharmacol. 14:475-480 (1992).

Vivian, B., *et al.*, Biotherapy 4:317-323 (1992).

Watson. J., *et al.*, J. Exp. Med. 150:849 (1979).

Weiden, P.L., *et al.*, "Graft-Versus-Host Disease in Allogeneic Marrow Transplantation," in Biology of Bone-Marrow Transplantation, Gale, R.P. and Fox, C.F., editors, Academic Press, New York, p. 37 (1980).

Williams, N.C., *et al.*, Clin. & Exp. Immunol., 87(2):183 (1992).

Wood, N.C., *et al.*, Clin. & Exp. Immunol., 87(2):183 (1992).

Yamamura, M., *et al.*, Acta Medica Okayama 44(6):301-308 (1990).

3. Background of the Invention

The immune system functions as the body's major defense against diseases caused by invading organisms. This complex system fights disease by killing invaders such as bacteria, viruses, parasites or cancerous cells. The immune system's ability to distinguish the body's normal tissues, or self, from foreign or cancerous tissue, or non-self, is an essential feature of normal immune system function. A second essential feature is memory, the ability to remember a particular foreign invader and to mount an enhanced defensive response when the previously encountered invader returns. The loss of recognition of a particular tissue as self and the subsequent immune response directed against that tissue produce serious illness.

An autoimmune disease results from the immune system attacking the body's own organs or tissues, producing a clinical condition associated with the destruction of that tissue. An autoimmune attack directed against the joint lining tissue results in rheumatoid arthritis; an attack against the conducting fibers of the nervous system results in multiple sclerosis. The autoimmune diseases most likely share a common pathogenesis and the need for safe and effective therapy.

Rheumatoid arthritis is one of the most common of the autoimmune diseases. Current treatments include three general classes of drugs

(Schumacher, 1988): anti-inflammatory agents (aspirin, non-steroidal anti-inflammatory drugs and low dose corticosteroids); disease-modifying antirheumatic drugs, known as "DMARDs" (antimalarials, gold salts, penicillamine, and sulfasalazine) and immunosuppressive agents (azathioprine, chlorambucil, high dose corticosteroids, cyclophosphamide, methotrexate, nitrogen mustard, 6-mercaptopurine, vincristine, hydroxyurea, and cyclosporin A). None of the available drugs are completely effective, and most are limited by severe toxicity.

In addition to their use in treating autoimmune conditions, immunosuppressive agents have also been used in treating or preventing transplantation rejection. Organ transplantation involving human organ donors and human recipients (allogeneic grafts), and non-human primate donors and human recipients (xenogeneic grafts), has received considerable medical and scientific attention (Roberts, 1989; Platt, 1990; Keown, 1991; Wang, 1991; Hasan, 1992; Murase, 1993). To a great extent, this effort has been aimed at eliminating, or at least reducing, the problem of rejection of the transplanted organ. In the absence of adequate immunosuppressive therapy, the transplanted organ is destroyed by the host immune system.

From follow-up studies on human transplant patients, as well as transplantation studies in animal model systems, the following features of transplant rejection have been established. The major targets in transplant rejection are non-self allelic forms of class I and class II major histocompatibility complex (MHC) antigens. Rejection is mediated by both antibodies and cytotoxic T lymphocytes (CTLs), with the participation of

CD4+ "helper" T cells (Noelle, 1991). In general, foreign class I MHC antigens stimulate CD8+ CTLs, and foreign class II MHC antigens stimulate CD4+ T cells (Roitt, 1991).

Another obstacle in transplantation, which has limited bone marrow transplants (BMT) in particular, is graft-versus-host disease (GVHD). GVHD is a condition in which transplanted marrow cells attack the recipient's cells (Thomas, 1975; Storb, 1984). Many BMT patients receiving HLA-identical marrow that tests negative in the mixed lymphocyte reaction (MLR) still develop GVHD, presumably because of a disparity between the recipient and donor at polymorphic non-HLA determinants. A large proportion of GVHD-afflicted individuals die as a result of GVHD (Weiden, 1980).

Presently, the most commonly used agents for preventing transplant rejection include corticosteroids, cytotoxic drugs that specifically inhibit T cell activation such as azathioprine, immunosuppressive drugs such as cyclosporin A, and specific antibodies directed against T lymphocytes or surface receptors that mediate their activation (Briggs, 1991; Kennedy, 1983; Storb, 1985; Storb, 1986). All of these drug therapies are limited in effectiveness, in part because the doses needed for effective treatment of transplant rejection may increase the patient's susceptibility to infection by a variety of opportunistic invaders, and in part because of direct toxicity and other side effects.

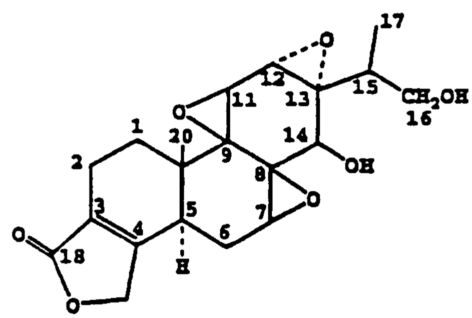
Cyclosporin A, currently the most effective and most commonly used agent, is significantly toxic to the kidney. This nephrotoxicity limits the quantity of drug that can be safely given. The physician is frequently forced to administer

sub-optimal doses of the drug because of this toxicity.

Accordingly, it would be desirable to have a drug capable of potentiating the action of immunosuppressive agents such as cyclosporin A. Ideally, such a drug would increase the efficacy of such immunosuppressive agents and also decrease deleterious side-effects by allowing administration of lower dosage levels.

4. Summary of the Invention

The invention relates to a purified compound for use in immunosuppressive therapy and referred to herein as 16-hydroxytriptolide, having the structural formula:



In one aspect, the invention includes a composition for use in immunosuppression therapy in a mammalian subject. The composition includes a therapeutically effective amount of purified 16-hydroxytriptolide in a pharmaceutically acceptable delivery vehicle, and also carried in the vehicle, an immunosuppressant drug, particularly cyclosporin A, FK506, azathioprine, methotrexate, rapamycin, mycophenolic acid, or a glucocorticoid. The composition has an increased immunosuppressive activity relative to the sum of the effects

produced by 16-hydroxytriptolide or immunosuppressant drug used alone, allowing greater immunosuppressive activity with reduced toxicity.

The composition is employed in immunosuppressive therapy, particularly in therapy for transplantation rejection and autoimmune disease.

In a preferred embodiment, the composition is used in treating transplantation rejection. In this regard, the composition can be used for treating rejection of an allograft or a xenograft, or complications caused by graft-versus-host disease. In a further embodiment, the composition is used in treating transplantation rejection, and the immunosuppressant drug is cyclosporin A.

Also disclosed is an immunosuppression therapy method for use in treating a mammalian subject. The method includes administering to the subject, a pharmaceutically effective amount of (i) purified 16-hydroxytriptolide and (ii) an immunosuppressant drug which is cyclosporin A, FK506, azathioprine, methotrexate, rapamycin, mycophenolic acid, a glucocorticoid, or a combination of two or more of such drugs. Use of the 16-hydroxytriptolide compound with the immunosuppressant drug results in a greater immunotherapeutic effect than expected from the effects of the triptolide compound or immunosuppressant agent when used alone.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Fig. 1 show a proposed structure of 16-hydroxytriptolide;

Fig. 2 shows an electron impact mass spectrum of 16-hydroxytriptolide;

Fig. 3 shows a fourier transform infrared (IR) spectrum of 16-hydroxytriptolide;

Fig. 4 shows a ^{1}H nuclear magnetic resonance (NMR) spectrum of 16-hydroxytriptolide;

Fig. 5 shows a proton-decoupled ^{13}C NMR spectrum of 16-hydroxytriptolide;

Fig. 6 shows a ^{1}H-^{1}H COSY spectrum of 16-hydroxytriptolide;

Fig. 7 shows a ^{1}H-^{13}C chemical shift correlation spectrum of 16-hydroxytriptolide;

Fig. 8 shows inhibition by 16-hydroxytriptolide of human peripheral blood lymphocyte (PBL) proliferation in the presence of anti-CD3 antibody;

Fig. 9 shows the suppressive effect of 16-hydroxytriptolide on IL-1 stimulation of mouse thymocyte proliferation;

Fig. 10 shows the suppressive effect of 16-hydroxytriptolide on IL-2 stimulation of HT-2 cell proliferation; and

Figs. 11A and 11B show the effect of 16-hydroxytriptolide on the concentrations of the cytokines IL-6 (Fig. 11A), TNFα (Fig. 11A), and IL-2 (Fig. 11B) in PHA-stimulated human PBL cultures.

Detailed Description of the Invention

I. Purified 16-Hydroxytriptolide

The invention employs a purified compound which may be isolated from *Tripterygium wilfordii*. The compound, designated "tripterygin" in parent application Ser. No. 08/058,321, is referred to

herein as "16-hydroxytriptolide." A postulated structural formula is shown in Fig. 1.

A. Purification of 16-Hydroxytriptolide

16-Hydroxytriptolide can be purified from the root xylem of *Tripterygium wilfordii*, a medicinal plant which is readily available in Fujiang Province and other southern provinces of China or through commercial sources in the United States. The presence of 16-hydroxytriptolide in the fractions generated at various stages of purification can be monitored by use of one or more of the assays described in Examples 3-6 (e.g., the PBL assay in Example 3). However, other compounds present in the mixture may also be detectable in the assays, thereby precluding unambiguous distinction of 16-hydroxytriptolide from such other compounds.

A protocol for isolating purified 16-hydroxytriptolide is described in Example 1. Briefly, dried plant material is ground into a crude powder and extracted by reflux with a volume of 95% ethanol that is about five times the weight (on a ml/g basis) of the dried plant material. The residual solid is extracted twice more with 95% ethanol, and the three resultant ethanol extracts are combined, filtered and reduced to a concentrated, syrupy extract (to about 2% of the original ethanol volume).

The concentrated extract is diluted by about 50% with water, filtered, and then extracted with several portions of methylene chloride. Following concentration, the collected methylene chloride fractions are applied to a silica gel column and eluted in a series of methylene chloride:methanol mixtures, i.e., mixtures with ratios of 100:0, 97:3, 95:5, and 90:10. The fractions which elute

with the 95:5 mixture are combined and concentrated.

The resultant concentrate is purified further by silica gel chromatography using a series of hexane:methylene chloride:methanol mixtures, i.e., mixtures having ratios of 1:2:5, 1:2:10, 1:2:15, and 1:2:20. The fractions which elute with the 1:2:15 mixture are collected and concentrated.

The resultant concentrate is purified further by silica gel chromatography using a series of hexane:
acetone mixtures, i.e., having ratios of 9:1, 8:2, 7:3, and 6:4. The fractions which elute with the 7:3 mixture are combined and concentrated.

The concentrate is purified by reversed phase (C-18) high performance liquid chromatography (HPLC) (70% methanol in water as eluant) by passage through two ODS columns as described in Example 1. The resultant material is crystallized from methanol to yield the final, purified 16-hydroxytriptolide.

B. Structural Analysis

Fig. 1 shows a structure and carbon atom numbering scheme for the 16-hydroxytriptolide compound of the invention.

Fast atom bombardment mass spectrometry of the 16-hydroxytriptolide compound revealed a molecular weight of 377.2 amu (MH^+) (Fig. 2). The exact mass was found to be 377.160050, in good agreement with the calculated value for $C_{20}H_{25}O_7$ (377.160028). These data are consistent with the structure shown in Fig. 1, having the molecular formula $C_{20}H_{24}O_7$ (mass M).

The infrared spectrum (Fig. 3) of 16-hydroxytriptolide includes a strong peak at 3426 cm^{-1} (hydroxyl), a small peak at 3030 cm^{-1} (epoxide C-

H), strong peaks at 1753 and 1676 cm^{-1} (α,β-unsaturated γ-lactone), and weak peaks at 908 and 830 cm^{-1} (epoxide C-C).

The ^{1}H-NMR spectrum (Fig. 4) of 16-hydroxytriptolide was as follows, where the assignments of ambiguous peaks were determined by COSY analysis (d_6-DMSO, δ-shifts in ppm): 4.82 (2H, m), assigned to the two protons at C-19; 3.88 (1H, d, J = 3 Hz), assigned to the proton at C-12; 3.64 (1H, d, J = 3 Hz), assigned to the proton at C-11; 3.32 (2H, m), assigned to the two protons at C-16; 3.24 (1H, d), assigned to the proton at C-14; 3.14 (1H, m), assigned to the proton at C-7; 2.60 (1H, d), assigned to the proton at C-5; 2.20 (1H, m), assigned to the α-proton at C-6; 2.15 (1H, m), assigned to the proton at C-15; 2.10 (2H, m), assigned to the two allylic protons at C-2; 1.82 (1H, t), assigned to the β-proton at C-6; 1.28 (2H, m), assigned to the two protons at C-1; 0.95 (3H, s), assigned to the three protons at C-20; and 0.83 (3H, d, J = 7 Hz), assigned to the three protons at C-17. Resonance peaks were found for all expected proton groups, and integration values were in agreement with those expected from the structure in Fig. 1.

Chemical shifts and assignments for selected resonance peaks in the proton-decoupled ^{13}C-NMR spectrum (Fig. 5) were as follows (d_6-DMSO, δ-shifts in ppm): 173.14, assigned to the carbonyl carbon (C-18); 162.44 and 123.01, assigned to the C=C carbons at C-4 and C-3, respectively; and 71.52 (C-14), 70.21 (C-19), 64.28 (C-13), 62.93 (C-9), 61.64 (C-8), 60.79 (C-7), 59.77 (C-16), 55.23 (C-12), and 54.53 (C-11), all corresponding to carbons singly bound to oxygen. The rest of the peaks, corresponding to carbons singly bound to carbon and/or hydrogen, were as follows: 40.33

(C-5), 38.67 (C-15), 35.20 (C-10), 28.99 (C-1), 22.62 (C-6), 16.60 (C-2), 13.70 (C-20), and 12.42 (C-17). Ambiguous peaks were determined from the ^{1}H-^{13}C COSY data below. In agreement with the molecular formula determined above, there were no other peaks in the ^{13}C spectrum.

The ^{1}H-^{1}H COSY spectrum, shown at Fig. 6, revealed coupling interactions between protons at C-11 and C-12, between protons at C-15, C-16, and C-17, between protons at C-5, C-6, and C-7, and between protons at C-1 and C-2.

The ^{1}H-^{13}C COSY spectrum (chemical shift correlation) shown at Fig. 7 revealed the shift correlations shown in Table 1 below. As expected, no crosspeaks were found for the resonance peaks assigned to carbon atoms which have no hydrogen substituents (carbons 3, 4, 8-10, 13, and 18).

Table 1

Carbon Assignment	^{13}C Chemical Shift (ppm)	^{1}H Chemical Shift (ppm)	Proton Assignment
1	28.99	1.28	1
2	16.60	2.10	2
5	40.33	2.60	5
6	22.62	2.20 1.82	6α 6ß
7	60.79	3.14	7
11	54.53	3.64	11
12	55.23	3.88	12
14	71.52	3.24	14
15	38.67	2.15	15
16	59.77	3.32	16
17	12.42	0.82	17
19	70.21	4.80	19
20	13.70	0.94	20

Taken together, the spectral data above are consistent with the structure shown in Fig. 1.

C. Compound Formulations

In the immunosuppressant therapy method of the invention, the purified 16-hydroxytriptolide compound may be administered with the immunosuppressant compound together in the same formulation, or separately in separate formulations. Where the compounds are prepared in separate formulations, the 16-hydroxytriptolide compound and immunosuppressant compound can be administered by different routes if desired.

The immunosuppressant drug which is administered with the 16-hydroxytriptolide compound is preferably one of the following:

(a) Cyclosporin A or cyclosporin C ("cyclosporin"), a non-polar cyclic oligopeptide;

(b)FK506, a fungal macrolide immunosuppressant;

(c) azathioprine, or 6[(1-Methyl-4-nitro-1H-immadazole-5yl)thio]1H-purine;

(d) methotrexate,

(e) rapamycin, a fungal macrolide immunosuppressant;

(f) mycophenolic acid, or 6-(1,3-Dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxy-5-isobenzofuranyl)-4-methyl-4-hexanoic acid; and

(g) an immunosuppressant glucocorticoid, such as prednisone or dexamethasone.

The proportions of the two components (16-hydroxytriptolide and immunosuppressant drug) are preferably in the range of 1:50 to 50:1 by weight.

When the purified 16-hydroxytriptolide and immunosuppressant compound are employed in the form of solid preparations for oral administration, the preparations may be tablets,

granules, powders, capsules or the like. In a tablet formulation, the compound is typically formulated with additives, for example, an excipient such as a saccharide or cellulose preparation, a binder such as starch paste or methyl cellulose, a filler, a disintegrator and so on, all being ones usually used in the manufacture of medical preparations.

For use in oral liquid preparation, the compounds may be prepared as a liquid suspension, emulsion, or syrup, being supplied either in liquid form or a dried form suitable for hydration in water or normal saline.

The compounds may be injected in the form of aqueous solutions, suspensions or oily or aqueous emulsions, such as liposome suspensions. Typically, for parenteral administration, the compounds are formulated as a lipid formulation, e.g., triglyceride, or phospholipid suspension, with the 16-hydroxytriptolide being dissolved in the lipid phase of the suspension.

II. Cytokine Inhibitory Activity

The purified 16-hydroxytriptolide compound was examined for immunosuppressive activity in a variety of *in vitro* biological assays.

A. Anti-Proliferative Effect on Human PBL's *in vitro*

One measure of immunosuppressive activity is suppression of stimulated peripheral blood lymphocyte (PBL) proliferation *in vitro*. In the assay detailed in Example 3, PBLs were activated *in vitro* by addition of anti- CD3 monoclonal antibody (X-35 antibody). At the same time, a solution of purified 16-hydroxytriptolide or solvent alone (control) was added to each culture, at selected concentrations. After 72 hours

incubation, tritiated thymidine was added to the culture medium, and thymidine incorporation into DNA was assayed, as a measure of DNA synthesis associated with cell proliferation.

Fig. 8 shows inhibition of peripheral blood lymphocyte proliferation, in the presence of stimulation with anti-CD3 antibody, as a function of the concentration of added 16-hydroxytriptolide. As seen, increasing amounts of purified 16-hydroxytriptolide produced dose dependent inhibition of proliferation of both unstimulated and anti-CD3-stimulated PBLs, with substantially complete inhibition occurring at a dose of 2×10^{-8} M 16-hydroxytriptolide. Half-maximal inhibition occurred at a 16-hydroxytriptolide concentration of approximately 10^{-8} M.

B. <u>Inhibition of IL-1 Action</u>

According to one important aspect of the invention, it has been discovered that 16-hydroxytriptolide has the ability to inhibit IL-1-induced lymphocyte proliferation. The implications of this finding, for use in immunosuppression therapy, are discussed in Section III below.

The ability of 16-hydroxytriptolide to suppress the cell- proliferative effect of IL-1 in mouse thymocytes, an index of IL-1 action (O'Gara), was examined (Example 4). In this study, mouse thymocytes in culture were stimulated with IL-1 in the presence of phytohemagglutinin (PHA) and increasing concentrations of purified 16-hydroxytriptolide. The cells were cultured for 72 hours, and during the last four hours, incubated with tritiated thymidine. Thymocyte proliferation was assessed by measurement of radiolabeled thymidine incorporation into DNA.

Fig. 9 shows the inhibition of IL-1-stimulated thymocyte proliferation in culture by 16-hydroxytriptolide. As seen in the figure, IL-1-stimulated cell proliferation was inhibited maximally by 16-hydroxytriptolide at a concentration of about 10^{-8} M, with half-maximal inhibition occurring at a concentration of about 3×10^{-9} M.

C. Inhibition of IL-2 Action

A similar study showed that 16-hydroxytriptolide also blocks the cell-proliferative activity of IL-2 on cultured HT-2 lymphocytes (O'Gara), as detailed in Example 5. Briefly, HT-2 cells were incubated in the presence of IL-2, and in the presence of increasing concentrations of 16-hydroxytriptolide. After 20 hours incubation, tritiated thymidine was added, and the incubation carried out for an additional 4 hours. Cells were harvested and counted as described for PBL proliferation.

Fig. 10 shows the inhibition of IL-2-stimulated thymocyte proliferation in culture by 16-hydroxytriptolide. As seen in the figure, the action of IL-2 on HT-2 cells was fully inhibited by 16-hydroxytriptolide at a concentration of about 10^{-7} M, with half-maximal inhibition occurring at a concentration of about 3×10^{-8} M.

D. Effect on Cytokine Production

The effect of 16-hydroxytriptolide on the production of the cytokines IL-1, IL-2, IL-6, and TNFα was assessed by measurement of the concentration of these cytokines in the medium of PHA-stimulated human PBL cultures. Cytokine levels were measured by standard ELISA methods using commercially available kits, as detailed in

Example 6. Briefly, assay buffer was added to each of the wells of a microtiter plate containing pre-bound anti-cytokine antibody, followed by addition of standard or sample solution, diluted appropriately for the cytokine concentration measured, followed by a second reporter-labeled antibody specific against the anti-cytokine antibody.

As shown in Fig. 11A, 16-hydroxytriptolide inhibited the production of Il-6 and TNFα. Production of these cytokines was suppressed to 1% of control concentrations in the presence of 10^{-7} M 16-hydroxytriptolide. Half-maximal inhibition occurred at about 6×10^{-9} M. As shown in Fig. 11B, 16-hydroxytriptolide inhibited production of IL-2 to about 10% of control at 10^{-7} M. Half maximal inhibition occurred at about 5×10^{-9} M. In contrast, 16-hydroxytriptolide did not alter IL-1 production by the cultured lymphocytes.

E. <u>Cytotoxicity</u>

Potential cytotoxicity of 16-hydroxytriptolide was assessed by measurement of the effect of 16-hydroxytriptolide on reduction of MTT (3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) by cultured cells, an index of cellular respiration and a sensitive assay for the detection of cytotoxicity (Green, *et al.*). Toxicity was evaluated *in vitro* in human PBLs and in mouse thymocytes and expressed as percent of control, as detailed in Example 7. Sodium azide was used as a cytotoxic control. Cytotoxicity was also assessed using the standard method of trypan blue dye staining. 16-hydroxytriptolide showed no significant toxicity at concentrations less than 10^{-7} M, the highest concentration range at which the compound produced dose-dependent inhibition of

cytokine production or action. Cytotoxicity was observed at higher concentrations. At 0.5×10^{-6} M, 16-hydroxytriptolide decreased cellular respiration by 29%, and at 2×10^{-6} M, 16-hydroxytriptolide decreased cellular respiration by 39%.

III. Treatment Method

The 16-hydroxytriptolide and immunosuppressant compounds of the invention are employed in immunosuppression therapy, particularly in treating transplantation rejection or autoimmune disease. According to an important feature of the invention, the effective dose of immunosuppressant drug is reduced significantly by co-administration of the drug with the purified 16-hydroxytriptolide compound, allowing higher drug doses to be administered and/or more prolonged treatment while reducing deleterious side effects.

Table 3 below gives a list of autoimmune diseases which are appropriate for immunotherapy.

Table 3

AUTOIMMUNE DISEASES	
Disease	Tissue Affected
Addison's disease	adrenal
Allergies	inflammatory cells
Asthma	bronchi
Atherosclerosis	vessel walls
Crohn's disease	intestine
Diabetes (Type I)	pancreas
Graves' disease	thyroid
Guillain-Barré Syndrome	nerve cells
Lupus erythematosus	multiple tissues
Multiple sclerosis	nerve cells

AUTOIMMUNE DISEASES	
Disease	Tissue Affected
Myasthenia Gravis	neuromuscular junction
Psoriasis	skin
Primary biliary cirrhosis	liver
Rheumatoid arthritis	joint lining
Uveitis	eye

In treatment of an autoimmune condition, the patient is given the 16-hydroxytriptolide and immunosuppressive compounds in a pharmaceutically acceptable vehicle or vehicles on a periodic basis, e.g., 1-2 times per week at a dosage level sufficient to reduce symptoms and improve patient comfort. Where the 16-hydroxytriptolide and immunosuppressant compounds are administered separately, the compounds may be administered with different dosing schedules as appropriate to maintain the potentiating effect of the 16-hydroxytriptolide compound on the immunosuppressant compound.

For oral administration, the 16-hydroxytriptolide compound may be given in liquid, tablet or capsule form, at a preferred dose of 0.1 and 2 mg/kg patient body weight per day. The dose may be increased or decreased appropriately depending on the response of the patient, and patient tolerance.

A parenteral suspension can be administered by injection, e.g., intravenously, intramuscularly, or subcutaneously, inhalation, or uptake via a mucosal membrane. A dose between about 0.05 and 1 mg 16-hydroxytriptolide/kg body weight per day is preferred, and this level may be increased or decreased appropriately, depending on the conditions of disease, the age of the patient,

and the ability of the patient to resist infection.

In general, it is preferred that 16-hydroxytriptolide is administered in an amount effective to achieve a serum concentration of 16-hydroxytriptolide of between about 10^{-8} and 10^{-7} M.

The dose of immunosuppressant drug that is administered is preferably 25-75% of the dose that would be administered when given in the absence of the 16-hydroxytriptolide compound, although lower levels of immunosuppressant drug may be administered.

For treating rheumatoid arthritis, the compounds may be administered by intravenous injection or by direct injection into the affected joint, for example. The patient may be treated at repeated intervals of at least 24 hours, over a several week period following the onset of symptoms of the disease in the patient.

For the treatment of systemic lupus erythematosus (SLE), as another example, the compounds may be administered by oral or parenteral administration, such as intravenous (IV) administration.

For therapy in transplantation rejection, the method is intended particularly for the treatment of rejection of heart, kidney, liver, and bone marrow transplants. The method is useful in the treatment of xenograft as well as allograft rejection. The method may also be used in the treatment of graft-versus-host disease, in which transplanted immune cells attack the allogeneic host. In addition, the composition may be administered chronically to prevent graft rejection, or in treating acute episodes of late graft rejection.

Treatment is typically begun perioperatively and is typically continued on a daily dosing regimen, for a period of at least several weeks, for treatment of acute transplantation rejection. During the treatment period, the patient may be tested periodically for immunosuppression level. Biopsy of the transplanted tissue may also be appropriate.

The following examples illustrate the method for obtaining purified 16-hydroxytriptolide and illustrate various physical, chemical, and *in vitro* properties of the compound. The examples are intended to illustrate, but in no way limit the scope of the invention.

<u>Example 1</u>

<u>Preparation of 16-Hydroxytriptolide</u>

A. <u>Bioassay</u>

Fractions produced at various stages in the purification procedure below were assayed to identify fractions containing 16-hydroxytriptolide by one or more of the assays described in Examples 3-6.

B. <u>Small-Scale Preparation of Crude Extract</u>

Tripterygium wilfordii plants were obtained in Taiwan or in Fujiang Province, China and were air-dried in sunlight. The root xylem of the plant (300 g) was ground into a crude powder and extracted with 1.5 l of 95% ethanol under reflux at 85°C for 4 hours. Following reflux, the residual crude powder was collected by filtration and extracted two more times as above using 900 ml 95% ethanol each time. The three ethanol extracts (total volume of about 3.3 l) were combined and the resulting mixture was concentrated at 50°C

under vacuum, to about 2% of the original volume, i.e., about 66 ml.

The concentrated mixture was then diluted with 33 ml water and filtered through Whatman #1 filter paper. The filtrate was extracted 4 times (50 ml/extraction) with methylene chloride. The combined filtrate (about 200 ml) was concentrated, and applied to a 6.5 cm (diameter) × 12 cm column of silica gel (~60-200 mesh). The column was washed successively with 600 ml methylene chloride, and 1500 ml methylene chloride:methanol (95:5). The fractions which eluted with the methylene chloride:methanol solvent were then concentrated under reduced pressure, yielding about 0.9 g of crude product (concentrate #1).

B. Further Purification of Extract

Forty grams of concentrate #1, prepared by scale-up of the procedure above, was applied to a 9 cm × 25 cm column of silica gel and eluted with the following series of methylene chloride:methanol mixtures (v:v): 100:0 (3 liters), 97:3 (4 liters), 95:5 (4 liters), and 90:10 (3 liters). The fractions which were eluted by the 95:5 mixture were concentrated under reduced pressure, yielding 12.9 g of concentrate #2.

Concentrate #2 was loaded on a 5.5 × 49 cm silica gel column and eluted with the following series of mixtures of hexane:CH_2Cl_2:methanol as follows: 1:2:5 (2 liters), 1:2:10 (2 liters), 1:2:15 (2 liters), and 1:2:20 (2 liters). Fractions which were eluted by the 1:2:15 mixture were concentrated under reduced pressure, yielding 2.26 g of a concentrate #3.

Concentrate #3 was purified by silica gel chromatography (2 cm × 42 cm) using the following

series of hexane:acetone mixtures: 9:1 (500 ml), 8:2 (1000 ml), 7:3 (1000 ml), and 6:4 (300 ml). Fractions which eluted with the 7:3 mixture were combined and concentrated, yielding 271 mg of a concentrate #4.

Concentrate #4 was purified by reversed phase HPLC on a 20 × 250 mm ODS column (Japanese Analytical, Inc., distributed by DyChrom, Santa Clara, CA). The mobile phase was 70% methanol in water, at a flow rate of 3 ml/min (100 mg/run). The elution time of 16-hydroxytriptolide was 3.6 min. 16-Hydroxytriptolide-containing fractions were concentrated under reduced pressure (75°C) and further purified by another ODS HPLC column (10 × 250 mm) using a mobile phase of 70% methanol in water, at a flow rate of 1 ml/min (20 mg/run). The elution time of 16-hydroxytriptolide was 2.5 min.

As a final step, purified 16-hydroxytriptolide was obtained by crystallization from methanol, yielding 13 mg of purified product.

Example 2

Physical Characteristics of 16-Hydroxytriptolide

Mass spectrometric analyses of 16-hydroxytriptolide were performed using a VG-ZAB2-EQ mass spectrometer for characterization by fast atom bombardment (FAB), and a Kratos MS-50 for high resolution mass determination using electron impact (EI) ionization for determination of exact mass values. FAB analysis (Fig. 2) revealed a molecular ion at m/z = 377.2 (MH^+). The exact mass found for MH^+ was 377.160050, in good agreement with the calculated value for $C_{20}H_{25}O_7$ (377.160028).

The infrared spectrum (Fig. 3) of 16-hydroxytriptolide (KBr pellet) was obtained using a Nicolet 510P FT-IR spectrometer.

NMR spectra (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C COSY) were obtained using a 300 MHz General Electric QE Plus spectrometer. All spectra were obtained in d_6-DMSO, with tetramethylsilane (TMS) included as a reference (defined as 0 ppm). The resultant spectra are shown at Figs 4-7 and described further in section I.B above.

Example 3

Suppression of PBL Proliferation *in Vitro*

A. Human Peripheral Blood Lymphocyte (PBL) Preparation

Human peripheral blood lymphocytes were prepared using an established method (Boyum). Human blood buffy coat samples, approximately 25 ml/donor, were obtained from the Stanford University Medical Center Blood Bank. Using sterile technique, the buffy coat samples were gently resuspended in a total volume of 100 ml with the addition of calcium and magnesium free Hank's balanced salt solution (HBSS, obtained from Gibco, Gaithersberg, MD) at room temperature. A volume of 25 ml of the cell suspension was then layered onto 15 ml of Ficoll-Pacque (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) in a 50 ml conical centrifuge tube. Tubes were centrifuged in a Beckman GPR tabletop centrifuge (GH-3.7 Rotor) at 400 × g for 30 minutes at 15°C.

Following centrifugation, the PBL suspensions at the interfaces were transferred to new 50 ml tubes using a transfer pipette, and the PBL samples were resuspended in a total volume of 45 ml HBSS and centrifuged at 354 × g for 10 minutes at 15°C. Supernatants were discarded. PBL's were resuspended in 10 ml HBSS, combined to make a total of 45 ml HBSS, and centrifuged at 265 × g for 10 minutes at 15°C. The cell pellets were suspended in 10 ml of X-Vivo tissue culture medium

(BioWhittaker Inc., Walkersville, MD) and counted using a hemocytometer. Tissue culture medium was then added to achieve a final cell concentration of 1×10^6 cells/ml. Additional dilutions were carried out as required for each assay.

Assays were carried out in 96 well sterile tissue culture plates (Costar 3790 and Costar 3595). A volume of 150 μl of X-Vivo medium or sterile distilled water was added to the outer wells of the plate to prevent evaporation of medium within the experimental wells. PBL's from 2 different donors were used in parallel in all experiments. A volume of 100 μl PBL suspension was added to each well using a multichannel pipette. Plates were incubated in an atmosphere of 93% air/7% CO_2 in a tissue culture incubator at 37°C. X-35 (AMAC #0178), an anti-CD3 surface antigen antibody, was used at 5 ng/ml to stimulate PBL proliferation.

16-Hydroxytriptolide was dissolved in ethanol or DMSO (10 mg/ml) and then diluted in sterile X-Vivo tissue culture medium to obtain the final concentrations required for each experiment.

Four hours prior to the conclusion of incubation, 50 μl of X-Vivo tissue culture medium containing 8 μCi/ml [^{3}H]Thymidine (49 Ci/mmol, Amersham, Arlington Heights, IL) was added to each tissue culture well. After 72 hours total incubation time, the cells were removed from the tissue culture wells and applied to filter paper using a cell harvester (Brandel, Model MB-24). The filter paper was dried for one hour under a heat lamp and then cut into 1 cm discs. Each sample was placed in a scintillation vial containing 2 ml of scintillation fluid (Biosafe, Research Products International Corp., Mount

Prospect, IL). Samples were counted in a Beckman LS 6000SC scintillation counter.

As shown in Fig. 8, 16-hydroxytriptolide inhibited PBL proliferation over a concentration range of 10^{-9} to 10^{-7} M. Half maximal inhibition occurred at a concentration of approximately 10^{-8} M.

Example 4

Inhibition of IL-1 Action on Mouse Thymocytes

Mouse thymocytes were prepared, and the action of IL-1, which stimulates thymocyte proliferation, was measured using standard techniques (O'Gara). Three to six week old C3H/HeN mice were obtained from Simonsen Laboratories, Gilroy, California and sacrificed by CO_2 inhalation. Thymi were removed, separated from adherent non-thymic tissue, homogenized in Hank's balanced salt solution (Gibco) using a glass homogenizer, and centrifuged at 180 × g for 10 minutes at 15°C. Following an additional wash in HBSS, the thymocytes were resuspended in RPMI 1640 tissue culture medium (Gibco) containing 50 µM 2-mercaptoethanol (Fisher, Orangeburg, NY), 2 mM glutamine (Gibco), 1 mM sodium pyruvate, non-essential amino acids, penicillin (100 U/ml) streptomycin (100 µg/ml), 10% heat-inactivated fetal bovine serum and Phytohemagglutinin (PHA, Pharmacia, final concentration 10 µg/ml). Cells were cultured in round-bottom 96 well microtiter tissue culture plates, 6×10^5 cells per well in a volume of 100 µl. 16-Hydroxytriptolide was diluted in tissue culture medium and added to the wells in the presence and absence of IL-1 (recombinant human IL-1ß, R&D Systems catalog # 201-LB, 0.1 ng/ml). Total volume was 150 µl per well.

Plates were incubated for 72 hours (95% air/5% CO_2, 37°C). During the last four hours of incubation, [^{3}H]- thymidine (Amersham, 49 Ci/mM) was added (0.5 μCi per well). Cells were harvested onto Whatman 934-AH glass microfiber filters and counted in a Beckman LS 6000 scintillation counter. Results were expressed as counts per minute per well.

Untreated cells showed minimal DNA synthesis (thymidine incorporation 80 cpm/well). PHA alone stimulated thymidine incorporation 2-3 fold. Treatment with 0.1 ng/ml IL-1 in the presence of PHA resulted in a 60-fold increase. Addition of the 16-hydroxytriptolide resulted in a dose-dependent inhibition of IL-1 stimulation, as shown in Figure 9. Inhibition was measured over a range of about 10^{-9} M to 2×10^{-6} M. Half-maximal inhibition occurred at 2×10^{-9} M.

Example 5

Inhibition of IL-2 Action by 16-Hydroxytriptolide

The effect of 16-hydroxytriptolide on the action of IL-2 was assessed by measurement of the compound's ability to inhibit IL-2-stimulated growth of the IL-2 dependent cell line HT-2, a well established biological assay of IL-2 action (O'Gara). HT-2 cells were cultured in 75 cm^2 (Corning, Corning, NY) tissue culture flasks in RPMI 1640 medium containing 10% fetal bovine serum (Hyclone, Logan, UT), 50 μM 2- mercaptoethanol (Fisher), 10 U/ml recombinant human IL-2 (Cetus, Norwalk, CT), 20 mM HEPES, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were passaged every 2 days. For experiments, HT-2 cells were centrifuged at 180 x g for 10 minutes, washed twice with 10 ml of IL-2- free culture medium and resuspended in RPMI 1640 medium

prepared as above, except containing 5% fetal bovine serum. Final cell concentration was 1 × 10^4/well. Cultures were incubated with varying concentrations of the 16-hydroxytriptolide (95% air/5% CO_2, 37°C) for 20 hours. Tritiated thymidine, 0.5 μCi/ well (Amersham, 49 Ci/mmol), was added and the incubation carried out for an additional 4 hours. Cells were harvested and counted as described for the PBL proliferation assay (Example 3).

As shown in Figure 10, 16-hydroxytriptolide, over a concentration range of 10^{-9} to 10^{-7} M, inhibited IL-2 induced DNA synthesis. Half maximal inhibition occurred at about 3 × 10^{-8} M.

Example 6

Effect of 16-Hydroxytriptolide on Cytokine Production

Human PBLs were prepared as in Example 3. The cells were incubated in the presence and absence of varying concentrations of 16-hydroxytriptolide, in the presence of 10 μg/ml PHA. Samples of tissue culture medium were collected following 24 hours incubation and stored at -70°C prior to assay.

Cytokine measurements were carried out using commercially available ELISA assay kits for cytokines IL-1, IL-2, IL-6, and TNFα (R&D Systems), in accordance with the supplier's protocols. In brief, 100 μl of the assay buffer supplied was added to each of the wells of a microtiter plate containing pre-bound anti-cytokine antibody, followed by 100 μl of standard or sample solution, diluted appropriately for the concentration range measured. All incubations were carried out at 37°C or 24°C, per the supplier's protocol.

Following two hours incubation, the plates were washed four times with assay buffer, and the second antibody, anti-anti-cytokine-horseradish peroxidase (HRP), was added to each well in a volume of 200 μl. Following another 2 hour incubation, the wells were washed four times with buffer, and 200 μl/well HRP substrate was added. After 20 minutes incubation, the reaction was terminated by the addition of 50 μl H_2SO_4 to each well. Optical density was determined using a Molecular Devices microtiter plate reader. Results were calculated as pg cytokine/ml medium.

As shown in Fig. 11A, 16-hydroxytriptolide effectively inhibited the production of IL-6 and TNFα. Production of these cytokines was suppressed to 1% of control levels in the presence of 10^{-7} M 16-hydroxytriptolide. Half-maximal inhibition occurred at about 4×10^{-9} M and 8×10^{-9} M, respectively. As shown in Fig. 11B, 10^{-7} M 16-hydroxytriptolide also decreased the production of IL-2 to about 10% of the level present in the untreated cultures. Half-maximal inhibition of IL-2 production occurred at about 4×10^{-9} M. In contrast, 16-hydroxytriptolide did not alter IL-1 production by the cultured lymphocytes.

Example 7

Evaluation of Potential Cytotoxicity

Potential cytotoxicity of 16-hydroxytriptolide was assessed by measurement of the compound's effect on the reduction of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) by cultured cells. MTT, a yellow-colored compound, is reduced by mitochondrial enzymes to form a purple crystalline reduction product (formazan), providing an index

of cellular respiration as well as a sensitive assay for cytotoxicity (Green, *et al.*).

Cytotoxicity was assessed in cultured human PBLs and mouse thymocytes. A stock solution of MTT (Sigma, St. Louis, MO), 5 mg MTT/ml phosphate buffered saline, pH 7.4, was prepared and stored in the dark at 4°C. Following 21 hours incubation under conditions identical to those above, 25 μl of MTT solution was added to each culture well. After an additional 3 hour incubation, the experiment was terminated by addition of a solution of 10% sodium dodecyl sulfate in 0.01 N HCl. Following overnight incubation at 37°C (to solubilize the formazan crystals, the MTT reduction product), optical density was determined at 570-650 nm in a Molecular Devices microtiter plate reader. Data are expressed as the ratio of the optical density of the 16-hydroxytriptolide-treated sample to that of untreated controls. No significant toxicity was observed at concentrations up to 10 $\mu g/ml$, the highest dose tested. The standard method of trypan blue vital dye staining was also used to assess toxicity. Results were consistent with those from the MTT reduction assay.

16-Hydroxytriptolide showed no significant toxicity at concentrations less than 10^{-7} M, the highest concentration range at which the compound produced dose-dependent inhibition of cytokine production or action. Cytotoxicity was observed at higher concentrations. At 0.5×10^{-6} M, 16-hydroxytriptolide decreased cellular respiration by 29%, and at 2×10^{-6} M, 16-hydroxytriptolide decreased cellular respiration by 39%.

Although the invention has been described with respect to particular methods and

applications, it will be appreciated that various changes and modifications may be made without departing from the spirit of the invention.

IT IS CLAIMED:

1. A composition for use in immunosuppression therapy in a mammalian subject, comprising

purified 16-hydroxytriptolide in a pharmaceutically acceptable delivery vehicle, and

also carried in the vehicle, an immunosuppressant drug selected from the group consisting of cyclosporin A, FK506, azathioprine, methotrexate, rapamycin, mycophenolic acid, and a glucocorticoid,

said composition having a potentiated immunosuppression activity with respect to a composition containing either the immunosuppressant drug or 16-hydroxytriptolide alone.

2. The composition of claim 1, for use in treating transplantation rejection in the subject.

3. The composition of claim 2, for treating rejection of an allograft.

4. The composition of claim 2, for treating rejection of a xenograft.

5. The composition of claim 2, for treating graft versus host disease.

6. The composition of claim 2, wherein the immunosuppressant drug is cyclosporin A.

7. An immunosuppression therapy method for use in a mammalian subject, comprising

administering to the subject (i) a pharmaceutically effective amount of an

immunosuppressant drug selected from the group consisting of cyclosporin A, FK506, azathioprine, methotrexate, rapamycin, mycophenolic acid, and a glucocorticoid; and (ii) purified 16-hydroxy-triptolide in an amount effective to potentiate the immunosuppressive effect of the immunosuppressant drug.

8. The method of claim 7, wherein the 16-hydroxytriptolide is administered orally in a pharmaceutically acceptable vehicle.

9. The method of claim 7, for use in treating transplantation rejection.

10. The method of claim 9, for use in treating rejection of an allograft.

11. The method of claim 9, for use in treating rejection of a xenograft.

12. The method of claim 9, for use in treating graft versus host disease.

13. The method of claim 9, wherein said administering is conducted over a several week period following transplantation.

14. The method of claim 13, wherein the immunosuppressant drug is cyclosporin A.

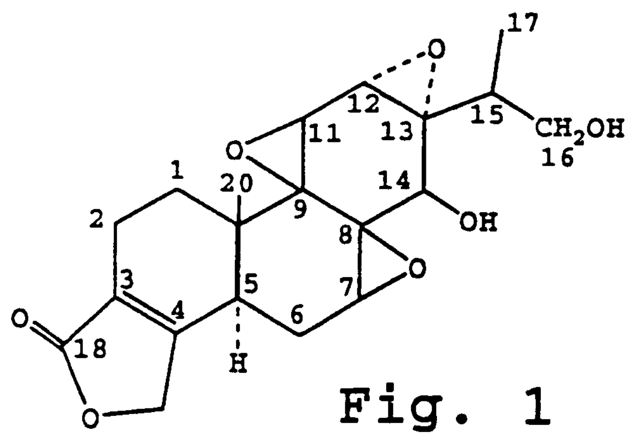
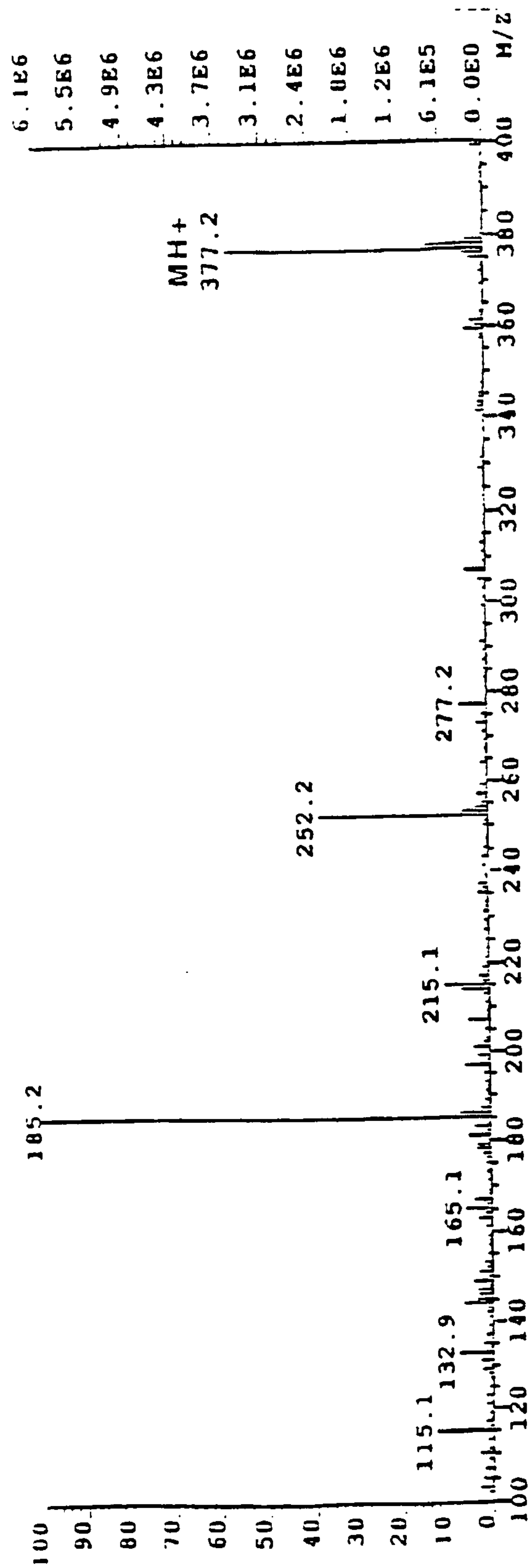


Fig. 1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277.2
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185.2
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4.3E6
3.7E6
3.1E6
2.4E6
1.8E6
1.2E6
6.1E5
0.0E0
M/z
100
90
80
70
60
50
40
30
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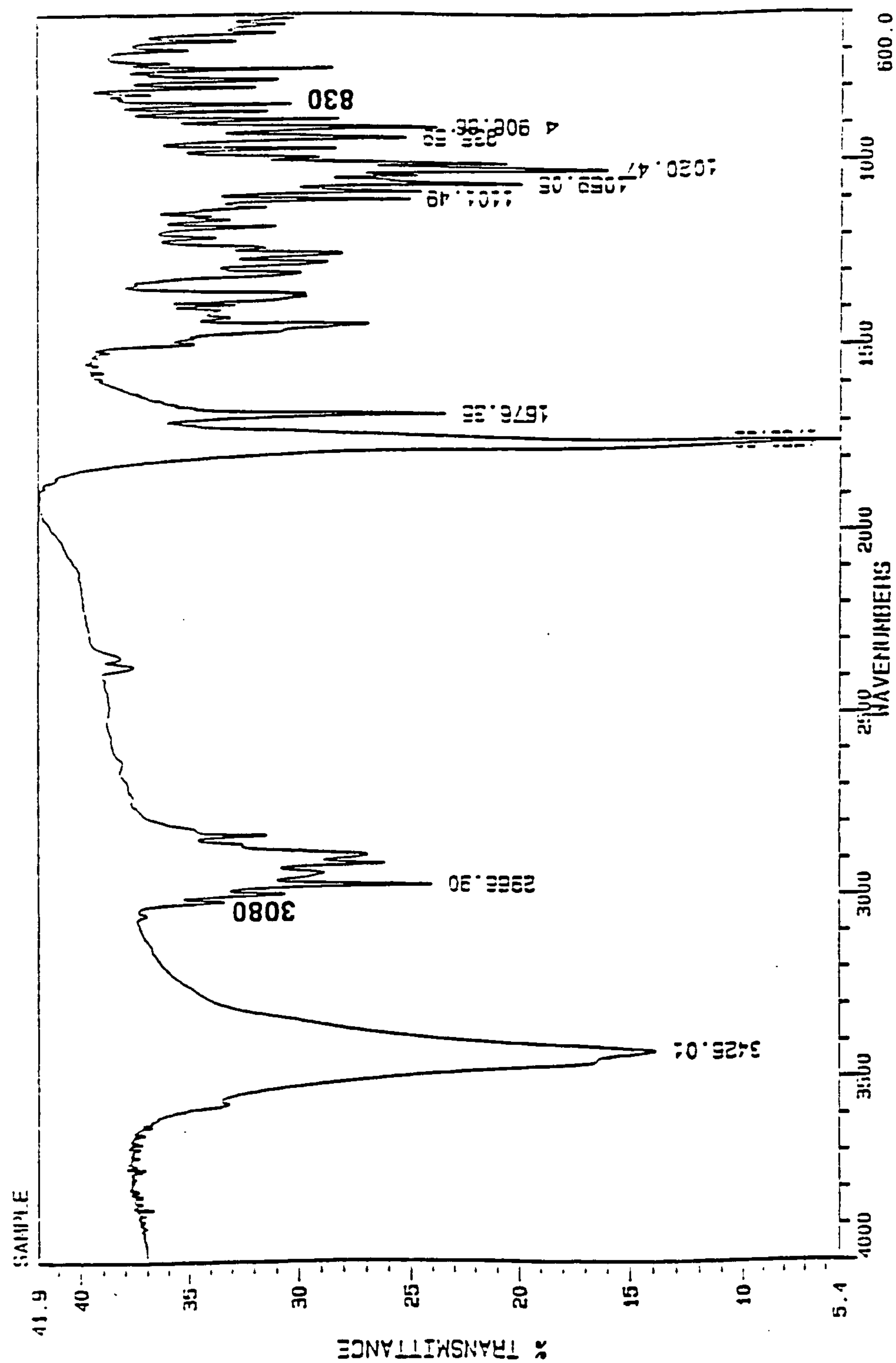
400

Fig. 2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1000
600.0

Fig. 3

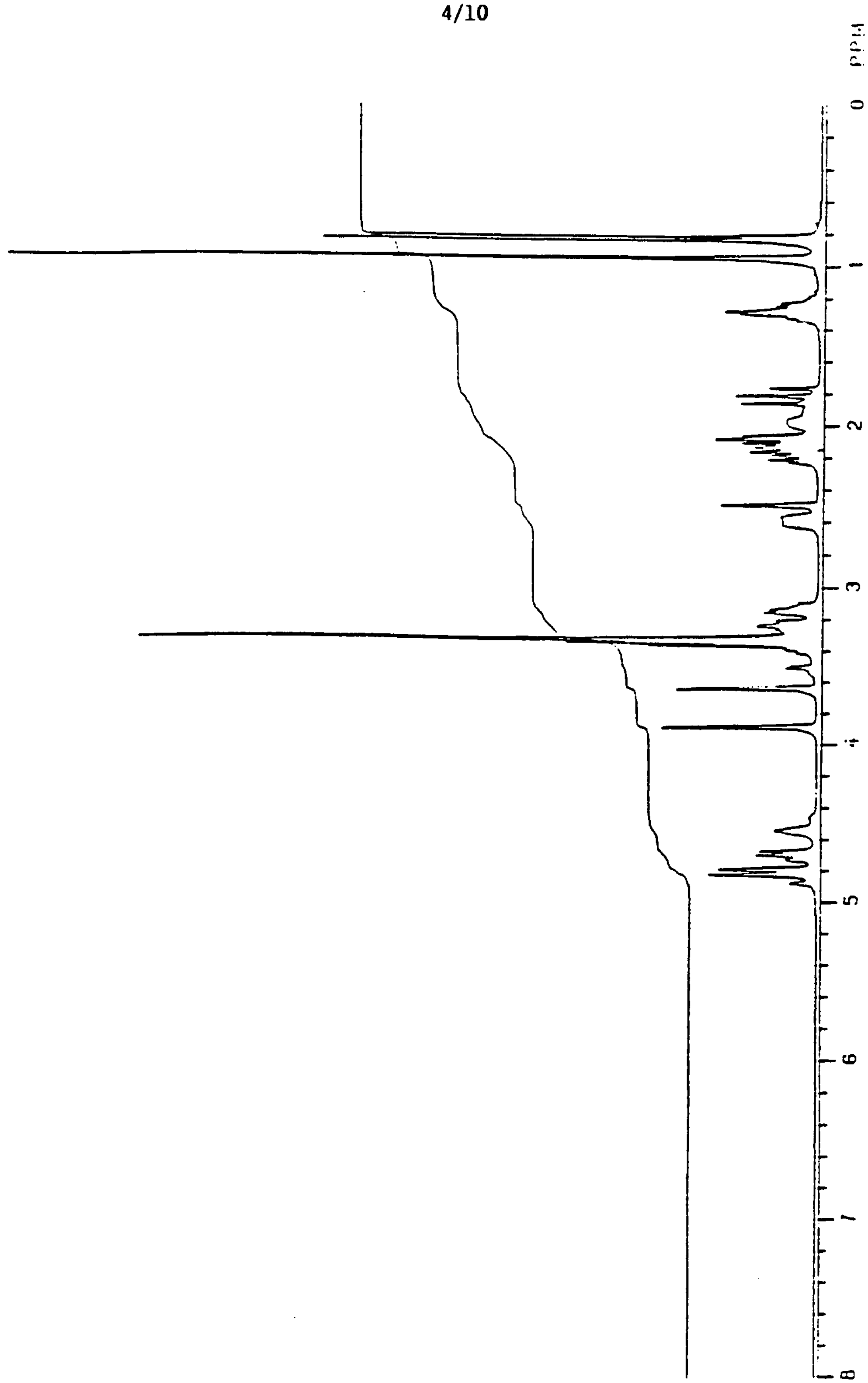


Fig. 4

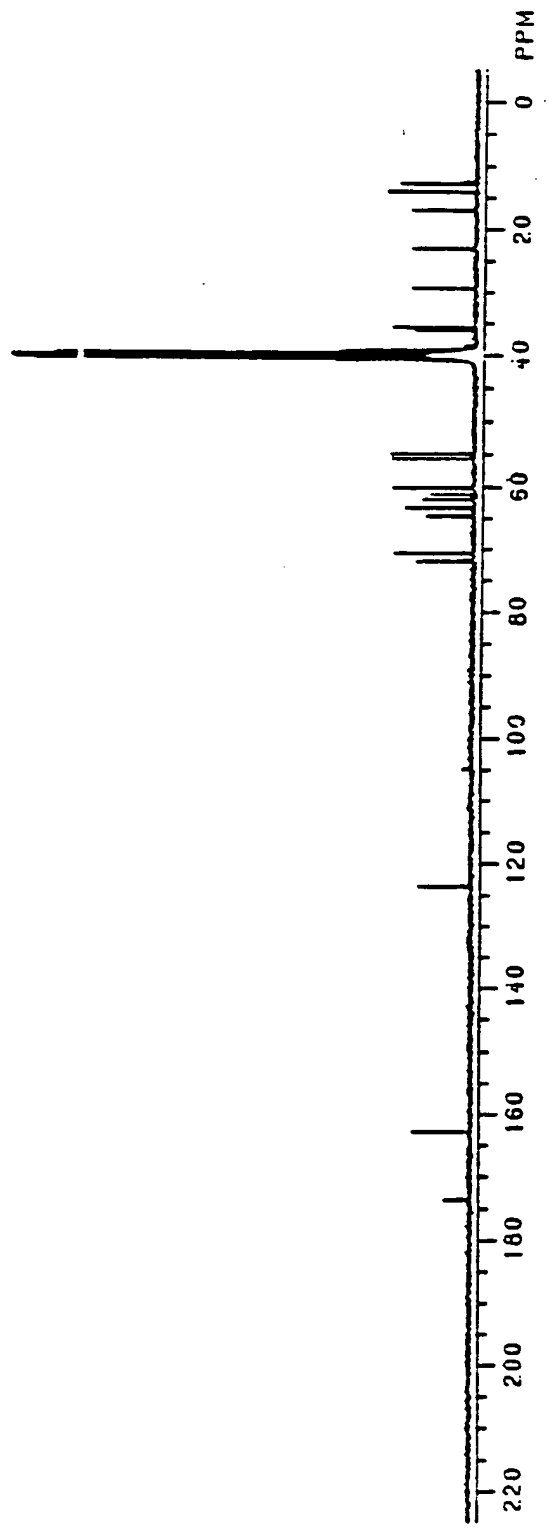


Fig. 5

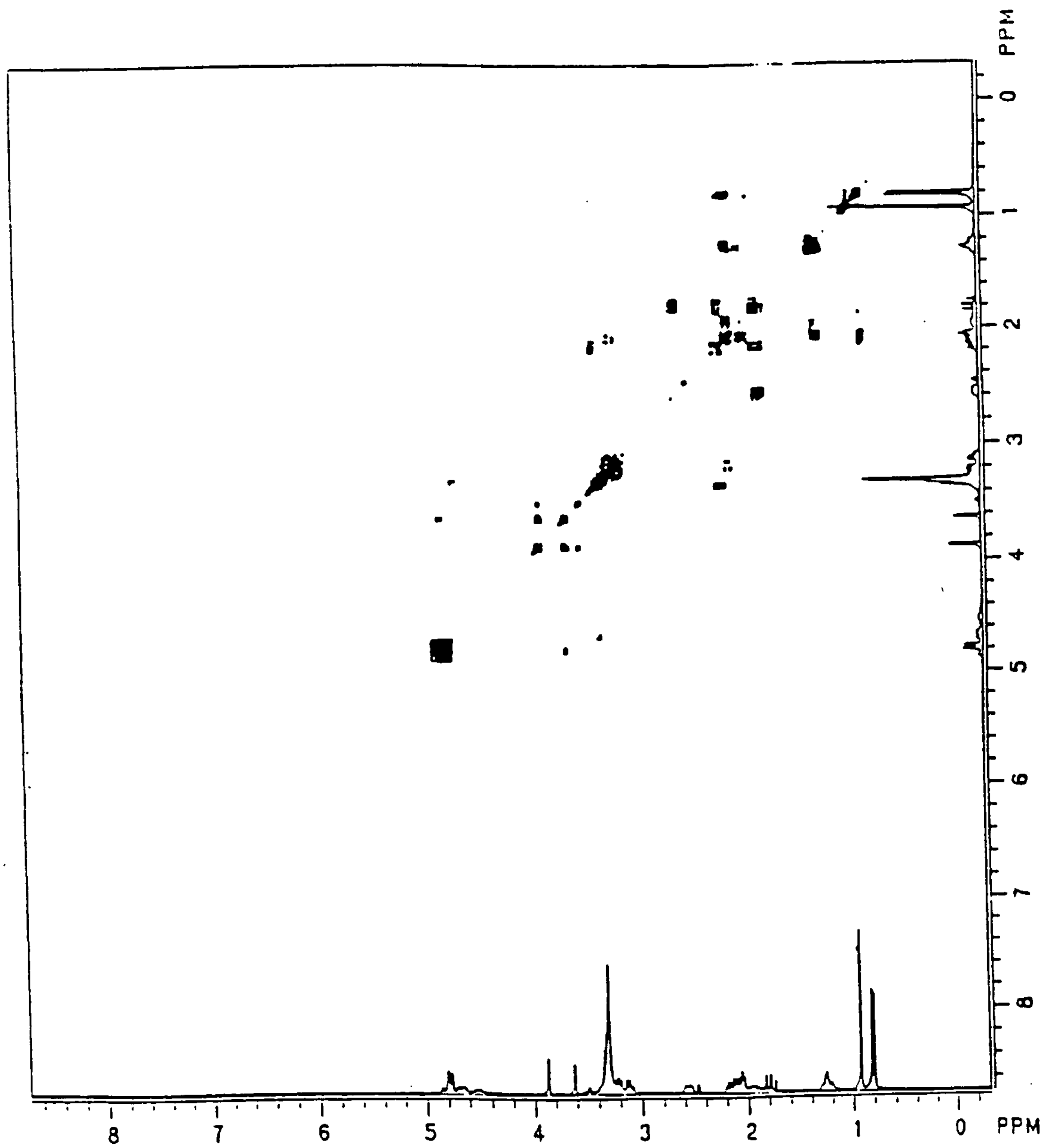


Fig. 6

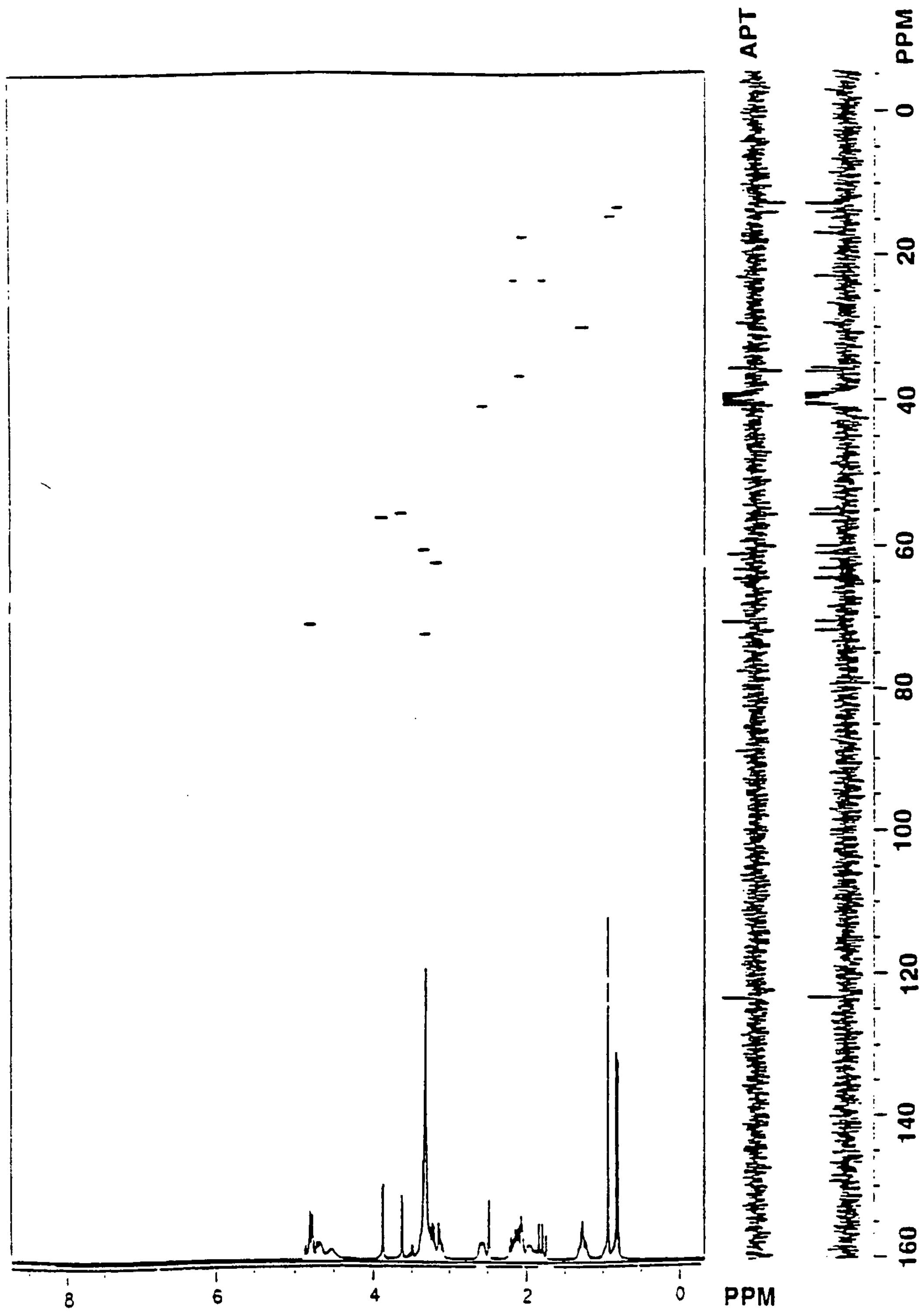
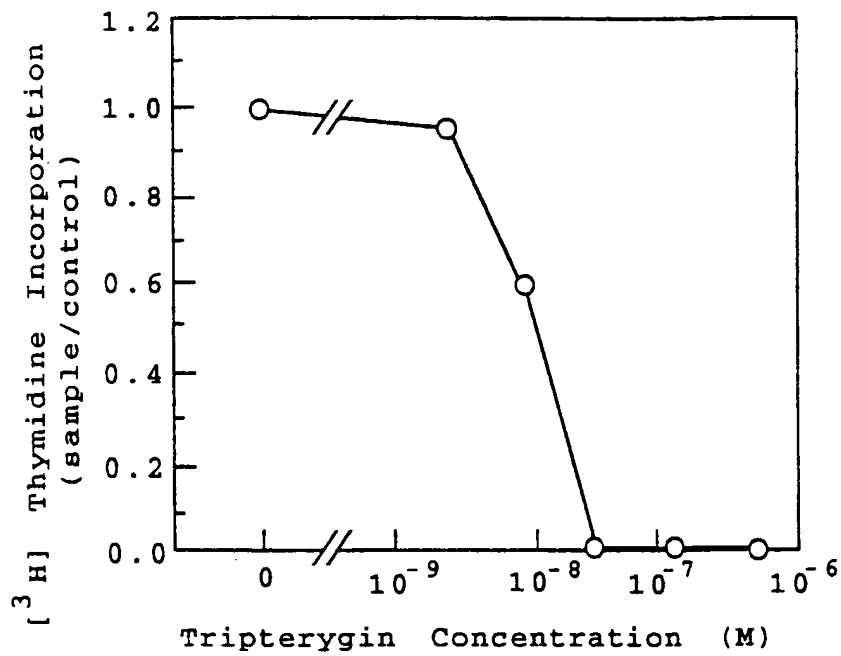
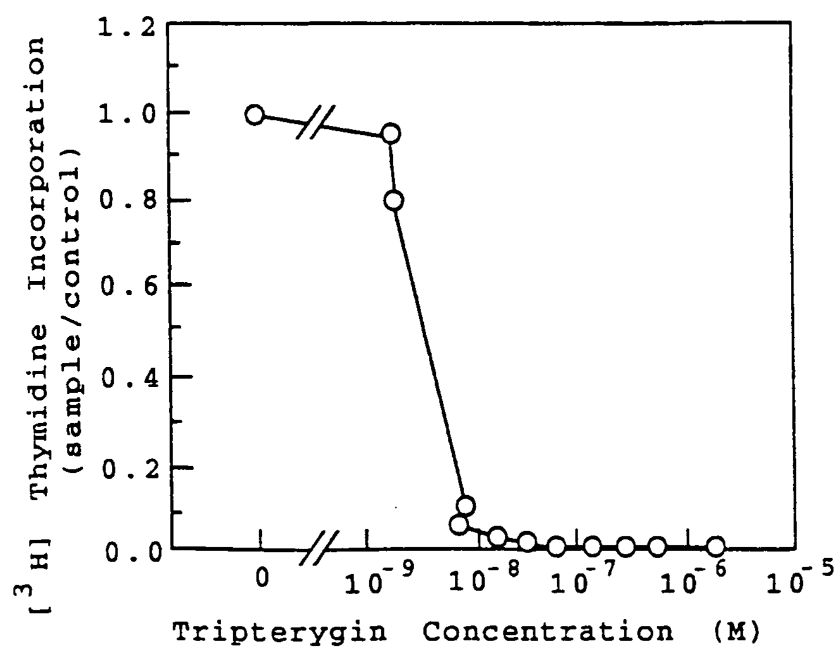


Fig. 7



1.2
1.0
0.8
0.6
0.4
0.2
0.0
[3 H] Thymidine Incorporation (sample/control)
0
10-9
10-8
10-7
10-6
Tripterygin Concentration (M)

Fig. 8



1.2
1.0
0.8
0.6
0.4
0.2
0.0
[3 H] Thymidine Incorporation (sample/control)
0
10-9
10-8
10-7
10-6
10-5
Tripterygin Concentration (M)

Fig. 9

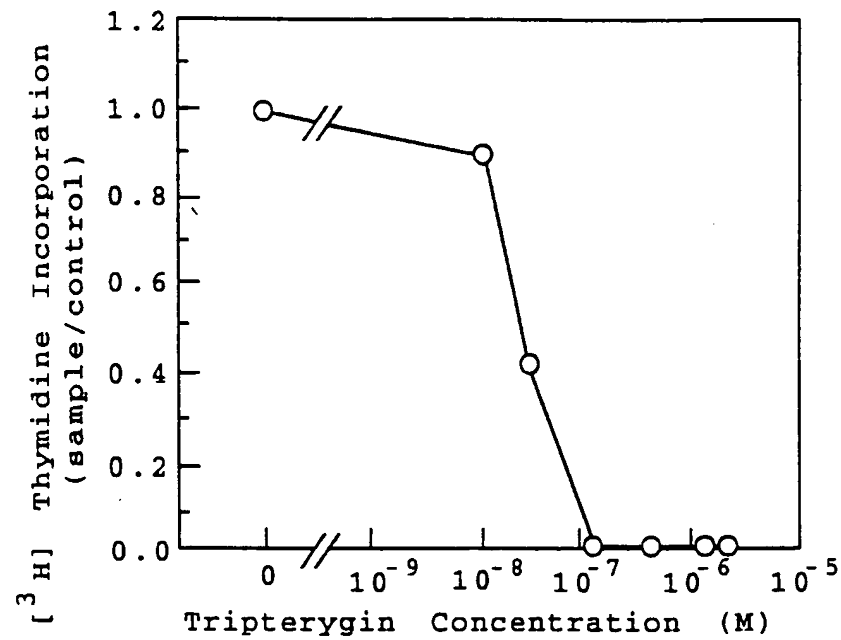

1.2
1.0
0.8
0.6
0.4
0.2
0.0
[^{3}H] Thymidine Incorporation (sample/control)
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Tripterygin Concentration (M)

Fig. 10

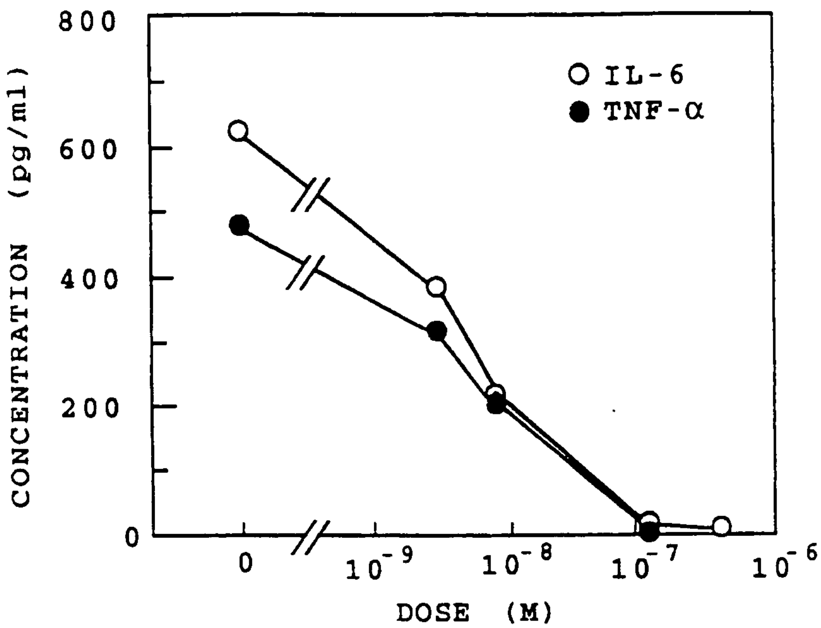


Fig. 11A

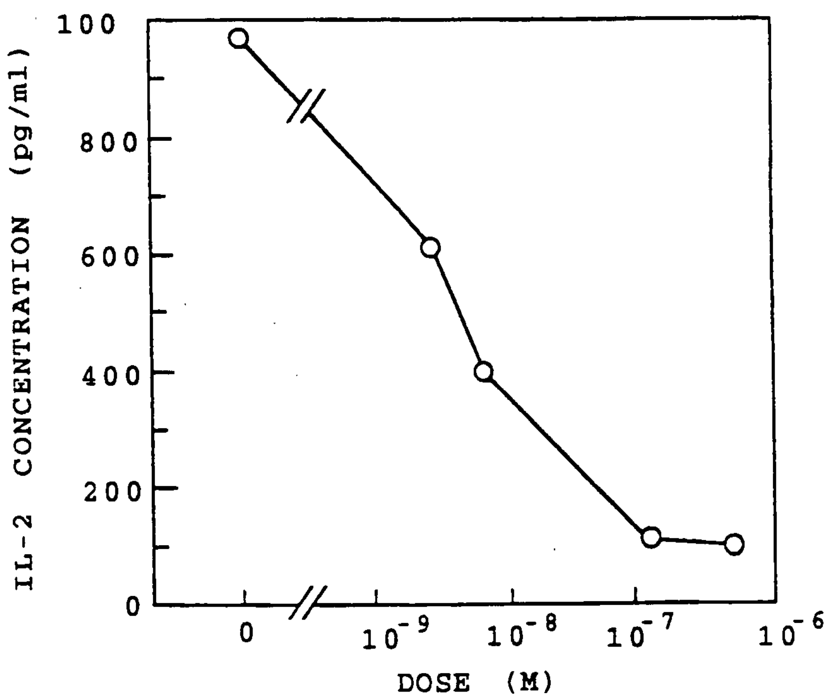


Fig. 11B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/04990

A. CLASSIFICATION OF SUBJECT MATTER IPC 5 A61K31/365 A61K37/02 A61K31/71 A61K31/52 A61K31/505 A61K31/57 //(A61K31/365,37:02,31:71,31:52,31:505,31:57)
According to International Patent Classification (IPC) or to both national classification and IPC
B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols) IPC 5 A61K
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INT. J. IMMUNOLOG. vol. 14, no. 6 , 1992 pages 963 - 969 YANG S., ET AL. 'Immunosuppression of triptolide and its effect on skin allograft survival' *see the whole document, esp. ultimate paragraph of the discussion * --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
8 August 1994	23.08.94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Isert, B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/04990

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 116, no. 15, 13 April 1992, Columbus, Ohio, US; abstract no. 143775f, page 72 ; see abstract & YAOXUE XUEBAO vol. 26, no. 10 , 1992 pages 759 - 763 MA P.C., ET AL. '16-Hydroxytriptolide: an new active compound from Tripterygium wilford H.' -----	1-14

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